Remarks

Claim Amendments:

Claims 1-28 and 125-130 are currently pending.

Claim 9 has been amended to clarify the terminology describing toxins.

No new matter has been added.

Filing Receipt

A review of Applicants' file indicates that Applicants' representative has yet to receive an updated filing receipt from the USPTO indicating the priority information of this application. The priority information was provided to the USPTO on the coversheet and transmittal of the originally filed non-provisional application, in a Preliminary Amendment filed November 30, 1999, and in the Declaration for Patent Application. Applicants hereby request an updated filing receipt indicating this priority information.

Information Disclosure Statement

Applicants' have not received a signed copy of the Information Disclosure Statement filed with the United States Patent and Trademark Office on November 10, 1999. Applicants' request the Examiner forward an initialed copy of the 1449 for Applicants' records. A duplicate copy and a copy of the stamped postcard are enclosed.

Rejection under 35 U.S.C. 112, first paragraph

Claims 1-10, 12 and 14-22, 24-28, and 125-130 are rejected under 35 U.S.C. 112, first paragraph because the specification "does not reasonably provide enablement for methods of administering any CpT (sic) motif containing oligonucleotide of less than 8 nucleotide residues including the elected species of 5' $X_1X_2CGX_3X_4$ 3' wherein X_1 is G, X_2 is T, X_3 is T, and X_4 is T for inducing a mucosal immunity to a recombinant peptide/polypeptide antigen within the context of therapeutic applications." The Examiner acknowledges that the specification enables a method for "inducing a mucosal immune response, comprising: administering to a mucosal surface of a subject an effective amount for inducing a mucosal immune response of an oligonucleotide having a length of at least 8 nucleotide residues and comprising CpG motif containing oligonucleotide including the elected species of 5' $X_1X_2CGX_3X_4$ 3' wherein X_1 is G,

X_2 is T, X_3 is T, and X_4 is T; and administering to the subject an antigen not encoded in a nucleic acid vector to the subject to induce a mucosal immune response."

In support of his rejection, the Examiner raises two main points. First, the Examiner states that claim 1 and claims dependent therefrom are not enabled because these claims fail to affirmatively recite a step of administering an antigen to the claimed subject. Second, the Examiner states that the claims are not enabled to the extent that they recite administration of the oligonucleotide by a route other than intranasal.

Applicants respectfully traverse the Examiner's first point. Claim 1 as currently pending recites a method for inducing a mucosal immune response comprising administering to a mucosal surface of a subject an effective amount for inducing a mucosal immune response of an oligonucleotide having a particular sequence and length, and exposing the subject to an antigen that is not encoded in a nucleic acid vector. Accordingly, the claim does affirmatively recite a step of exposing the subject to an antigen. The term "exposing the subject to an antigen" intends to embrace both passive and active exposure to the antigen, as evidenced by dependent claims 2 and 10 and as described in the specification on page 27, lines 14-26. The specification teaches that the subject can be exposed to the antigen by direct administration by any route including but not limited to oral, intranasal and intratracheal routes, or by entry of a foreign pathogen into the body as an example of passive exposure.

Applicants have taught in sufficient detail in the specification how to expose a subject to an antigen. Furthermore, co-pending US Patent Application 09/241,653, cited in the IDS filed on November 10, 2001, demonstrates that the subject may be contacted with the antigen up to 30 days after administration of the CpG oligonucleotide to produce an antigen specific immune response. The evidence provided in the co-pending application is consistent with the teaching in the instant specification that the subject may be "exposed to an antigen".

Applicants respectfully traverse the Examiner's second point. The specification adequately describes methods for carrying out routes of administration, other than intranasal, that will effect a mucosal immune response. There is no specific teaching in any of the references cited by the Examiner to demonstrate that one of skill in the art would not believe applicants assertion that administration of a CpG oligonucleotide by any route including but not limited to intranasal, ocular, vaginal, rectal or inhalation would produce a mucosal immune response to antigen. Furthermore, a 2001 publication (McCluskie and Davis, Vaccine 19, p. 413-422,

attached hereto as Exhibit 1 and submitted in an IDS filed herewith) demonstrates that CpG administration in combination with an antigen by routes other than intranasal, including intrarectal produce mucosal immunity. Thus, the teachings of this post-filing reference is consistent with the teachings of the invention. The specification as filed adequately enabled practice of the invention by administration of CpG oligonucleotides via mucosal routes other than intranasal.

In view of the foregoing, Applicants respectfully request that the Examiner reconsider and withdraw the rejection under 35 U.S.C. § 112, first paragraph.

Rejection under 35 U.S.C. 102(b)

In view of Krieg 6,218,371

Claims 1-10, 12, 14-22, 24-28, and 125-130 are rejected under 35 U.S.C. 102(b) as being anticipated by Krieg (6,218,371), as evidenced by Moldoveanu et al., Sato et al., McCluskie et al., and Mestecky et al.

According to the Examiner, Krieg et al. teaches oral and nasal administration of an oligonucleotide and/or an antigen, CpG motifs including 5' GTCpGTT 3', the use of a colloidal dispersion system, the use of a cytokine including B-7 as an adjuvant in combination with a CpG containing oligonucleotide of at least 8 nucleotides, and IL-6 production. The Examiner relies on the remaining references as factual evidence that CpG motifs when administered to the mucosal surface of a subject generate a mucosal immunity, and that local production of IL-6 at the mucosal surface stimulates production of IgA antibodies. The Examiner concludes that Krieg et al. would inherently generate mucosal immunity.

Applicants previously submitted a Declaration under 37 C.F.R. 1.131 stating that the claimed invention was conceived of and reduced to practice prior to the effective filing date of USP 6,218,371. The Examiner has not taken the Declaration into consideration because the factual evidence was not submitted to the PTO. Applicants submit herewith a supplemental Declaration under 37 C.F.R. 1.131 describing corroborative support. In view of the supplemental Declaration and corroborative support, USP 6,218,371 is no longer prior art to the instant application.

The remaining references are moot as to their evidentiary weight because the Krieg reference does not anticipate the claimed invention. For purposes of clarity on the record,

Applicants herby note that each of the Moldoveanu et al., Sato et al., and McCluskie et al. have effective priority/publication dates after the effective priority date of the instant patent application (May 22, 1998). Moldoveanu et al., was published in July of 1998 (dated abstract attached hereto as Exhibit 2). McCluskie et al. was published in November of 1998 (dated abstract attached hereto as Exhibit 3). Sato et al has an effective US filing date of July 1998.

In view of the foregoing, Applicants respectfully request that the Examiner reconsider and withdraw the rejections under 35 U.S.C. § 102(b) and 102(e).

Rejection under 35 U.S.C. 103(a)

Claims 1-10, 12, 14-22, 24-28, and 125-130 are rejected under 35 U.S.C. 103(a) as being unpatentable over Krieg et al. (USP 6,218,371), taken with Krieg et al. (*Trends in Microbiology* 6(1):23-27, 1998) as evidenced by Moldoveanu et al., Sato et al., McCluskie et al. and Mestecky et al. According to the Examiner, although the Krieg reference does not teach the use of Th2 response inducing adjuvants (i.e., alum), Krieg et al. (*Trends in Microbiology*) teaches that alum is effective as a Th2 response inducing adjuvant. The Examiner concludes that it would have been obvious for one of ordinary skill to have used alum in the immunization methods of the Krieg reference. The Examiner further states that "it is well established that an mucosal immunity is same as Th2 response." Applicants respectfully traverse the rejection for the reasons stated below.

Applicants traverse the Examiner's assertion that it is well established that mucosal immunity is the same as a Th2 response. A Th response is not defined by the site of induction but rather by the cytokines present while T and B cells are developing antigen specific responses; examples of Th1 cytokines being INF-γ and IL-12 and Th2 cytokines being IL-4 and IL-5. It is possible to induce either Th1 or Th2 responses by either systemic or mucosal routes. The data of the instant invention clearly demonstrate that CpG oligonucleotides induce a mucosal immune response of a Th1 type. The instant specification teaches that Cholera toxin is a mucosal adjuvant that induces a Th2 immune response. Accordingly, a mucosal immune response is not synonymous with a Th2 immune response.

Applicants have asserted that their date of invention is earlier than the effective filing date of Krieg et al. USP 6,218,371, thereby removing this reference as prior art. Additionally, the secondary reference (Krieg et al. Trends in Microbiology) does not provide the deficiencies

of the primary Krieg reference. In particular, the secondary reference does not teach that mucosal immunity can be achieved upon mucosal administration of CpG oligonucleotides. The reference does not teach administration of CpG oligonucleotides to a subject in need of a mucosal immune response or administration of a CpG oligonucleotide in an amount effective to induce a mucosal immune response (as evidenced by the production of IgA). The secondary reference also does not teach formulation of CpG oligonucleotides for ocular administration, vaginal administration, rectal administration, intranasal administration or inhalation. Further, as described above Moldoveanu et al., Sato et al., McCluskie et al. have publication/effective US priority dates after the priority date of the instant patent application.

In view of the foregoing, Applicants respectfully request that the Examiner reconsider and withdraw the rejection under 35 U.S.C. § 103(a).

Rejection under 35 U.S.C. 103(a)

Claims 1-10, 12, 14-22, 24-28, and 125-130 are rejected under 35 U.S.C. 103(a) as being unpatentable over Briles et al (USP 6,042,838) taken with either Krieg (WO96/02555) or Krieg et al. (USP 6,194,388) and Hoades et al. According to the Examiner,

Briles et al describe a vaccine that includes an antigen (PspA of *S. pneumoniae*) that stimulates a mucosal immune response when applied to a mucosal surface. According to Briles the antigen may be formulated with an adjuvant. CpG oligonucleotides are not listed as adjuvants. Krieg et al has been cited for the teaching that CpG oligonucleotides are adjuvants. The Krieg references, however, do not disclose the finding of the instant invention that CpG oligonucleotides produce a mucosal immune response when administered to a subject with an antigen that does not by itself produce a mucosal immune response, or that CpG oligonucleotides augment a mucosal immune response when administered to a subject with an antigen that does by itself produce a mucosal immune response. In view of this, one of skill in the art would not use a CpG oligonucleotide as an adjuvant for producing or augmenting a mucosal immune response in the absence of an antigen that produces a mucosal immune response on its own.

Hoades was cited for the teaching that IFN production is associated with enhancing a Th1 response and suppressing Th2 stimulation. According to the Examiner, one of skill in the art would have recognized that CpG oligonucleotides produce a Th1 immune response in view of the teachings of Hoades. It is unclear to applicants why this is relevant to the claimed invention,

because production of a Th1 immune response is not claimed. Additionally, on page 598 of Hoades, first paragraph it is taught that since Il-4 and Il-5 are cytokines produced by Th2 cells that "it might be expected that the antibodies resulting from interaction of B cells with Th2 cells would express selectively enhanced IgG1, IgA, and IgE components in comparison to the responses supported by Th1 cells." This teaching suggests that a Th1 producing adjuvant such as CpG oligonucleotides might not produce a mucosal immune response.

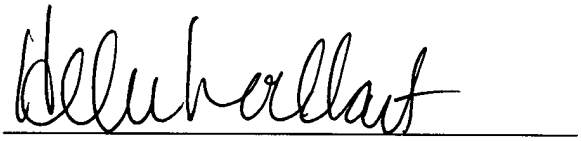
Thus, the combination of references does not render the claimed invention obvious.

Summary

Applicants believe that each of the pending claims is in condition for allowance. Applicants respectfully request that the Examiner telephone Applicants' agent in the event that the claims are not found to be in condition for allowance.

If the Examiner has any questions and believes that a telephone conference with Applicants' agent would prove helpful in expediting the prosecution of this application, the Examiner is urged to call the undersigned at (617) 720-3500 (extension 266).

Respectfully submitted,



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Docket No. C1040.70006US00
Date: November 20, 2003
x11/23/03

Exhibit 1

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Oral, intrarectal and intranasal immunizations using CpG and non-CpG oligodeoxynucleotides as adjuvants

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Abstract

We have previously demonstrated that synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs (CpG ODN) are potent adjuvants in mice when delivered by intramuscular, intranasal and subcutaneous routes. Herein, using tetanus toxoid (TT) as a model antigen in BALB/c mice, we compared the ability of CpG ODN to induce mucosal and systemic humoral immune responses when antigen was delivered by three different routes: intrarectal, intranasal and oral. Results showed differences in immune responses with the three routes and also revealed that non-CpG "control" ODN had adjuvant effects when used at mucosal sites. This was unexpected since non-CpG ODN do not have such immunostimulatory effects in vitro or after parenteral immunization. These findings were further investigated after oral delivery of a killed influenza vaccine on its own as well as combined with TT and hepatitis B surface antigen. Our findings demonstrate that with mucosal delivery, there is a Th2 immunostimulatory effect associated with the phosphorothioate ODN backbone, and that the presence of CpG motifs shifts this towards a Th1 response.

Keywords: Adjuvant; Mucosal; CpG

1. Introduction

The primary local defense mechanism at the mucosal surfaces of the gastrointestinal (GI), genitourinary (GU) and respiratory tracts is the common mucosal immune system (CMIS). It can be divided into inductive sites, where antigens are encountered, endocytosed and presented to B and T cells, and effector sites where antigen-specific B and T cells reside and perform their respective functions to protect mucosal surfaces [1,2]. The principal inductive sites are the bronchus- and nasal-associated lymphoid tissues (BALT, NALT) in the respiratory tract, and the gut-associated lymphoid tissue (GALT) in the GI tract, which are commonly targeted by intranasal (IN) and oral immunizations respectively. However, there is also a significant rectal-associated lymphoid tissue (RALT) in the large intestine, and a number of successful rectal immunizations have been reported [3–6].

Mucosal vaccines generally require the use of adjuvants. Bacterial toxins, such as cholera toxin, are commonly used as mucosal adjuvants in animal models [7,8], however, toxicity prevents their use in humans. A new class of adjuvant is CpG DNA, which contains unmethylated CpG dinucleotides in particular base contexts (CpG motifs) and which is most often given in the form of synthetic oligodeoxynucleotides (CpG ODN). CpG ODN provides a broad adjuvant effect including: stimulation of B cells to proliferate, secrete immunoglobulin (Ig), cytokines (IL-6, IL-12), and to be protected from apoptosis [9–11]; enhancement in

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PII: S0264-410X(00)00208-5

expression of class II MHC and B7 costimulatory molecules [12,13]; and direct activation of monocytes, macrophages and dendritic cells to secrete various cytokines and chemokines [11,14]. Stimulatory CpG motifs are typically preceded on the 5′ side by an ApA, GpA or GpT dinucleotide and followed on the 3′ side by two pyrimidines, especially TpT [10]. It has previously been demonstrated that ODN that do not contain CpG motifs (non-CpG ODN) do not have stimulatory effects in vitro nor do they have an immunostimulatory effect at typical adjuvant doses by parenteral routes (and only a small effect can be found with very high doses) [12].

To date, most work using CpG ODN as adjuvant has been with parenteral immunization of mice and, more recently, non-human primates [12,15–20]. However, several studies have now shown that IN delivery using CpG ODN as an adjuvant results in strong systemic and mucosal immune responses to coadministered antigens including hepatitis B surface antigen (HBsAg) [21,22], β-galactosidase [23] and whole killed influenza virus [24].

A number of studies have shown that the route of mucosal immunization can influence both the strength and nature of immune responses [25–27]. In addition, certain mucosal adjuvants (e.g., different mutants of *Escherichia coli*) will elicit distinct immune response profiles when delivered by different routes [28]. To date, there have been no comparative studies using CpG ODN as adjuvant with different mucosal routes. We have evaluated the strength and nature of systemic and mucosal immune responses induced in mice after administration of tetanus toxoid (TT) with CpG ODN by three different mucosal routes: oral, IN, and intrarectal (IR). Results revealed differences in responses with the three routes, and also revealed that non-CpG "control" ODN had adjuvant effects when used at mucosal sites. Therefore, we also examined the effects of CpG and non-CpG ODN on immune responses after oral delivery with other antigens, namely a killed split influenza virus vaccine on its own as well as combined with TT and HBsAg.

2. Materials and methods

2.1. Mice

All experiments were carried out using female BALB/c mice aged 6–8 weeks with 5–10 mice per experimental or control group. For all immunizations, mice were lightly anaesthetized with Halothane® (Halocarbon Laboratories, River Edge, NJ).

2.2. Antigens

Plasma-derived HBV S protein (HBsAg, *ad* subtype, Genzyme Diagnostics, San Carlos, CA), formalin-inactivated tetanus toxoid (TT, Aventis Pasteur, Swiftwater, PA), and/or trivalent influenza virus vaccine (A/Sydney/5/97, A/Beijing/262/95, B/Harbin/7/94, FLUVIRAL®, Biochem Vaccines, Laval, QC).

2.3. Adjuvants

CpG ODN sequence #1826 (5′-TCCATGA*CG*TTCCTGA*CG*TT-3′) as well as non-CpG ODN sequences #1982 (5′-TCCAGGACTTCTCTCAGGTT-3′) and #2138 (5′-TCCATGAGCTTCCTGAGCTT-3′) were each synthesized with a nuclease-resistant phosphorothioate backbone (Hybridon Speciality Products, Milford, MA). LPS level in ODN was undetectable (< 1 ng/mg) by Limulus assay (Whittaker Bioproducts, Walkersville, MD). Cholera toxin (CT) was obtained from Sigma (St. Louis, MO).

2.4. Immunization of mice

Each animal was immunized at 0, 7 and 14 days with one or more antigens, alone or combined with an adjuvant in saline (0.15 M NaCl), by one of three different routes. The antigens and doses were TT (10 μg), HBsAg (10 μg) and/or FLUVIRAL® (50 μl, equivalent to 1/10 human dose and containing 1.5 μg hemagglutinin [HA] for each of A/Sydney/5/97, A/Beijing/262/95, and B/Harbin/7/94 viral strains). CpG and non-CpG ODN adjuvants were used at doses of 1, 10 or 100 μg. For comparative purposes, cholera toxin (CT, 1 or 10 μg), which is a well-established mucosal adjuvant, was used as a positive control.

For oral immunization, antigen (TT, FLUVIRAL, or a TT/FLUVIRAL/HBsAg combination), with and without adjuvant, made up to a total volume of 50 μl was administered using a 1 cc tuberculin syringe (Becton Dickinson, Franklin Lakes, NJ) attached to a 20-gauge olive tip steel feeding tube (Fine Science Tools, North Vancouver, BC), which was passed down the oral cavity and esophagus.

For IN immunization, TT alone or with adjuvant, in a total volume of 5–20 μl, was applied as droplets over the external nares of the mouse.

For IR immunization, TT alone or with adjuvant in a total volume of 20 μl was deposited into the rectum via the anus using a 200 μl pipette tip.

2.4.1. Collection of samples

All samples were collected over a two-day period, which was 1 week ± 1 day after the third and final immunization. Plasma and fecal pellets were collected and lung washes carried out as previously described

[21]. Vaginal secretion samples were collected by washing the vaginal cavity three times with 75 μl (225 μl total) of PBS containing 0.1 μg sodium azide (Sigma, St. Louis, MO). Gut washes were obtained by removing the small intestine and passing 200 μl PBS containing 0.1% sodium azide through each of three 10-cm sections. All samples were stored at −20°C until assayed by ELISA.

2.4.2. Evaluation of immune responses

Ag-specific antibodies in the individual samples were detected and quantified by end-point dilution ELISA assay as described previously for IgG, IgG1, IgG2a [12] and IgA [21] isotypes. Titers for IgG isotypes in plasma, and IgA in mucosal samples were defined as the highest sample dilution that resulted in an absorbance value (OD_{450}) at least twice that of non-immune plasma or mucosal sample. Ag-specific antibody titers for a group of animals were expressed as geometric mean titers ± the standard error of the mean (GMT ± SEM) of individual animal values, which were themselves the average of triplicate assays.

2.4.3. Statistical analysis

Data were analyzed using the GraphPAD InStat program (GraphPAD Software, San Diego). The statistical significance of the difference between groups was calculated using transformed data ($\log_{10}$) of ELISA titers, by Student's 2-tailed *t*-test for two groups, or by 1-factor analysis of variance (ANOVA) followed by Tukey's test for three or more groups. Differences were considered to be not significant with $p > 0.05$.

3. Results

3.1. CpG ODN administered with TT at different mucosal sites: Adjuvant effect on plasma IgG

To evaluate the adjuvant activity of CpG ODN at different mucosal sites, TT was administered by three routes of delivery (oral, IR, IN). Without adjuvant, TT induced no or only very low (< 10) levels of anti-TT IgG antibodies in the plasma of mice in the IR or IN groups and low to medium titers in most animals with the oral route (Fig. 1, panels (a–c)). Addition of CpG ODN to TT greatly enhanced the response rate and titers for all routes of delivery. The ranking of anti-TT plasma IgG was oral > IN > IR ($p < 0.05$) with a low dose of CpG (1 μg). At larger doses (10 or 100 μg) equivalent

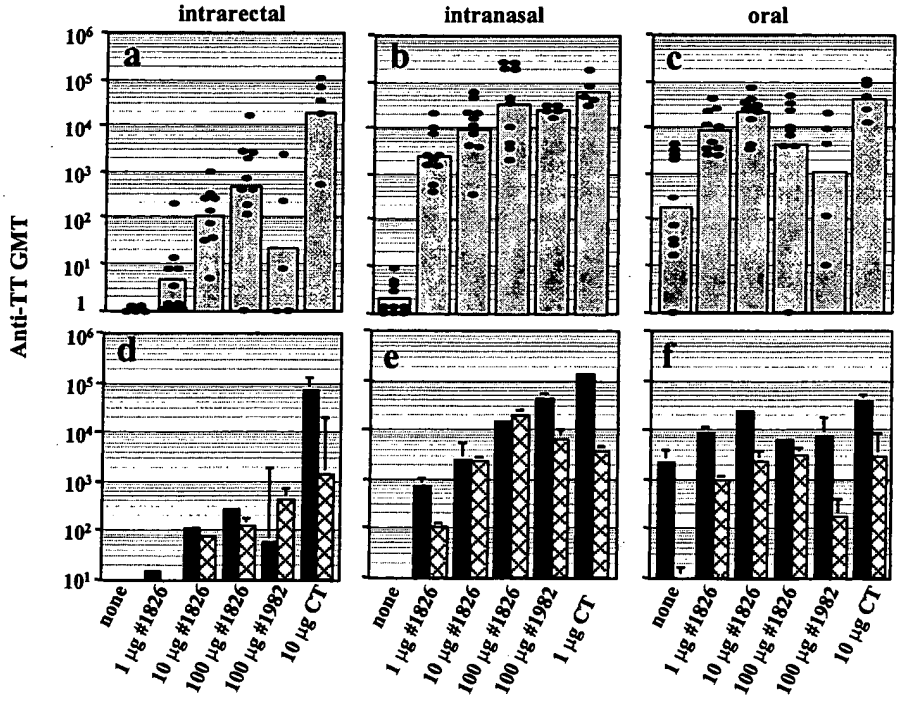

Fig. 1. BALB/c mice were immunized on days 0, 7 and 14 by IR, IN, or oral delivery of 10 μg TT either alone, with a CpG ODN (#1826, 1, 10 or 100 μg), a non-CpG ODN (#1982, 100 μg) or cholera toxin (CT, 1 or 10 μg) as adjuvant. *Upper panels* (a–c): Each point represents the titer for TT-specific antibodies (anti-TT IgG GMT) in individual animals in plasma taken 1 week after final immunization. *Lower panels* (d–f): Each bar represents the group geometric mean (±SEM) of the titer for TT-specific antibodies (anti-TT IgG GMT) of IgG1 (black bars) or IgG2a (hatched bars) isotypes in plasma taken 1 week after final immunization.

responses were seen for oral and IN groups ($p > 0.05$), and these were higher than the IR group (≤ 0.002) (Fig. 1, panels (a–c)). For oral delivery, mean IgG titers were enhanced nearly 100-fold compared to TT alone, with similar effects being obtained with all doses of CpG ODN used (1, 10 or 100 μg, $p > 0.05$) (Fig. 1(c)). With IN delivery, where TT alone resulted in 6/10 with no anti-TT and only 4/10 with very low titer, the addition of CpG ODN gave high titers in all animals (30/30), and there was a significant ODN dose-response ($p < 0.05$) (Fig. 1(b)). The adjuvant effects of CpG ODN were less obvious with the IR route, but there was also a strong dose-response for the ODN with each 10-fold increase in CpG ODN dose giving 10-fold higher anti-TT titers ($p < 0.05$), suggesting that perhaps optimal effects were not realized, even with the 100 μg dose (Fig. 1(a)).

Compared to CT, which was used at commonly reported doses (10 μg for oral and IR, 1 μg for IN), CpG ODN (10 or 100 μg doses) induced equivalent levels of anti-TT IgG for oral and IN routes ($p > 0.05$), but lower levels with the IR route (Fig. 1).

Non-CpG ODN (100 μg dose), which was included to be a negative control, had an unexpected adjuvant effect, with at least some of the mucosal routes. All IN mice (5/5), most oral mice (3/5) but only one IR mouse (1/5) developed good IgG titers (> 1000). This adjuvant effect of non-CpG ODN was unexpected since the same non-CpG ODN (#1982) did not have stimulatory effects on mouse cells in vitro, nor had it been found to have adjuvant effects when given by a parenteral route (intramuscular injection) [12]. A previous IN study had also not detected a non-CpG ODN effect, albeit at a 10-fold lower ODN dose (10 μg) than used in this study [21].

It should be noted that the adjuvant effects of non-CpG ODN on plasma IgG were inferior to those of the same dose of CpG ODN for oral and IR routes. Another difference between the two classes of ODN adjuvants was the Th bias of the IgG response. As an indirect way to determine Th-bias of induced responses, IgG isotypes were measured (Th1: IgG1 < IgG2a, Th2: IgG2a < IgG1). When IgG titers were high enough to detect isotypes (i.e. after oral delivery), TT alone induced a strong Th2 response with virtually no IgG2a. CpG ODN, which has been shown to be a Th1-type adjuvant, was able to overcome the strong Th2 bias of TT and gave a mixed IgG1/IgG2a response with all routes (Fig. 1, panels (d–f)). Furthermore, with IN and oral delivery, the proportion of IgG2a antibodies increased with higher doses of CpG ODN. In contrast, TT-specific antibody responses with both non-CpG ODN (#1982) or CT as adjuvant remained strongly Th2 (IgG1 ≫ IgG2a) biased. Thus, despite the finding that both CpG and non-CpG ODN had an adjuvant effect on TT-specific IgG responses (at the doses tested), the strong Th1 bias typically seen with CpG ODN was not seen with non-CpG ODN.

3.2. CpG ODN administered with TT at different mucosal sites: Adjuvant effect on IgA response

Assays were carried out to detect IgA at mucosal surfaces of the respiratory (lung and nasal wash), digestive (saliva, gut, feces) and reproductive (vagina) systems. After administration of TT alone by IR, IN or oral routes, none or only very low levels of TT-specific IgA were detected in the various mucosal samples recovered from the mice (Fig. 2). In contrast, when CpG ODN was added, it was possible to induce significant levels of IgA in most mice, although this was somewhat dependent upon the route of immunization and dose of CpG ODN. For example, with IR delivery, dose-dependent levels of IgA were detected in the gut wash and feces, but no IgA was found in lung, nasal, vaginal or saliva samples. On the other hand, a TT-specific IgA response was induced at all (6/6) or most (4/6) of the mucosal surfaces tested, with IN or oral delivery respectively. The strength of these IgA responses were dose-dependent for IN but not for oral delivery. Only two of six assays for the IR group had detectable IgA (Fig. 2).

The finding of IgA at multiple mucosal surfaces indicates that the CMIS was activated. Nevertheless, certain surfaces appeared to be preferential for a given route. For example, more mice in the IR group had anti-TT IgA (titers > 5) in the feces (8/30) than the gut (4/30), whereas more mice in the oral group had IgA in gut (16/30) than the feces (5/30). With IN delivery, most mice (11/15) had IgA in nasal washes, but this was not seen after IR or oral immunizations. These results suggest that a stronger mucosal response occurs at the site of antigen uptake than elsewhere (i.e., GALT, RALT and NALT for oral, IR, and IN routes, respectively).

TT-specific IgA titers with CpG ODN were generally as high as or higher than those obtained with CT. Non-CpG ODN was not as potent as CpG ODN for induction of IgA, but it was still possible to obtain significant IgA titers in most mucosal samples after IN, but not IR or oral immunization.

3.3. CpG and non-CpG ODN administered orally with influenza vaccine

Since the non-CpG control ODN (sequence #1982) had an unexpected immunostimulatory effect with mucosal delivery of TT, in the experiments described above, we examined whether similar immunostimulatory effects would also be seen by adding a different non-CpG ODN (sequence #2138) to TT and other

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Oral, intrarectal and intranasal immunizations using CpG and non-CpG oligodeoxynucleotides as adjuvants

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Abstract

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Keywords: Adjuvant; Mucosal; CpG

1. Introduction

The primary local defense mechanism at the mucosal surfaces of the gastrointestinal (GI), genitourinary (GU) and respiratory tracts is the common mucosal immune system (CMIS). It can be divided into inductive sites, where antigens are encountered, endocytosed and presented to B and T cells, and effector sites where antigen-specific B and T cells reside and perform their respective functions to protect mucosal surfaces [1,2]. The principal inductive sites are the bronchus- and nasal-associated lymphoid tissues (BALT, NALT) in the respiratory tract, and the gut-associated lymphoid tissue (GALT) in the GI tract, which are commonly targeted by intranasal (IN) and oral immunizations respectively. However, there is also a significant rectal-associated lymphoid tissue (RALT) in the large intestine, and a number of successful rectal immunizations have been reported [3–6].

Mucosal vaccines generally require the use of adjuvants. Bacterial toxins, such as cholera toxin, are commonly used as mucosal adjuvants in animal models [7,8], however, toxicity prevents their use in humans. A new class of adjuvant is CpG DNA, which contains unmethylated CpG dinucleotides in particular base contexts (CpG motifs) and which is most often given in the form of synthetic oligodeoxynucleotides (CpG ODN). CpG ODN provides a broad adjuvant effect including: stimulation of B cells to proliferate, secrete immunoglobulin (Ig), cytokines (IL-6, IL-12), and to be protected from apoptosis [9–11]; enhancement in

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expression of class II MHC and B7 costimulatory molecules [12,13]; and direct activation of monocytes, macrophages and dendritic cells to secrete various cytokines and chemokines [11,14]. Stimulatory CpG motifs are typically preceded on the 5′ side by an ApA, GpA or GpT dinucleotide and followed on the 3′ side by two pyrimidines, especially TpT [10]. It has previously been demonstrated that ODN that do not contain CpG motifs (non-CpG ODN) do not have stimulatory effects in vitro nor do they have an immunostimulatory effect at typical adjuvant doses by parenteral routes (and only a small effect can be found with very high doses) [12].

To date, most work using CpG ODN as adjuvant has been with parenteral immunization of mice and, more recently, non-human primates [12,15–20]. However, several studies have now shown that IN delivery using CpG ODN as an adjuvant results in strong systemic and mucosal immune responses to coadministered antigens including hepatitis B surface antigen (HBsAg) [21,22], β-galactosidase [23] and whole killed influenza virus [24].

A number of studies have shown that the route of mucosal immunization can influence both the strength and nature of immune responses [25–27]. In addition, certain mucosal adjuvants (e.g., different mutants of *Escherichia coli*) will elicit distinct immune response profiles when delivered by different routes [28]. To date, there have been no comparative studies using CpG ODN as adjuvant with different mucosal routes. We have evaluated the strength and nature of systemic and mucosal immune responses induced in mice after administration of tetanus toxoid (TT) with CpG ODN by three different mucosal routes: oral, IN, and intrarectal (IR). Results revealed differences in responses with the three routes, and also revealed that non-CpG "control" ODN had adjuvant effects when used at mucosal sites. Therefore, we also examined the effects of CpG and non-CpG ODN on immune responses after oral delivery with other antigens, namely a killed split influenza virus vaccine on its own as well as combined with TT and HBsAg.

2. Materials and methods

2.1. Mice

All experiments were carried out using female BALB/c mice aged 6–8 weeks with 5–10 mice per experimental or control group. For all immunizations, mice were lightly anaesthetized with Halothane® (Halocarbon Laboratories, River Edge, NJ).

2.2. Antigens

Plasma-derived HBV S protein (HBsAg, *ad* subtype, Genzyme Diagnostics, San Carlos, CA), formalin-inactivated tetanus toxoid (TT, Aventis Pasteur, Swiftwater, PA), and/or trivalent influenza virus vaccine (A/Sydney/5/97, A/Beijing/262/95, B/Harbin/7/94, FLUVIRAL®, Biochem Vaccines, Laval, QC).

2.3. Adjuvants

CpG ODN sequence #1826 (5′-TCCATGA*CG*TTCCTGA*CG*TT-3′) as well as non-CpG ODN sequences #1982 (5′-TCCAGGACTTCTCTCAGGTT-3′) and #2138 (5′-TCCATGAGCTTCCTGAGCTT-3′) were each synthesized with a nuclease-resistant phosphorothioate backbone (Hybridon Speciality Products, Milford, MA). LPS level in ODN was undetectable (< 1 ng/mg) by Limulus assay (Whittaker Bioproducts, Walkersville, MD). Cholera toxin (CT) was obtained from Sigma (St. Louis, MO).

2.4. Immunization of mice

Each animal was immunized at 0, 7 and 14 days with one or more antigens, alone or combined with an adjuvant in saline (0.15 M NaCl), by one of three different routes. The antigens and doses were TT (10 μg), HBsAg (10 μg) and/or FLUVIRAL® (50 μl, equivalent to 1/10 human dose and containing 1.5 μg hemagglutinin [HA] for each of A/Sydney/5/97, A/Beijing/262/95, and B/Harbin/7/94 viral strains). CpG and non-CpG ODN adjuvants were used at doses of 1, 10 or 100 μg. For comparative purposes, cholera toxin (CT, 1 or 10 μg), which is a well-established mucosal adjuvant, was used as a positive control.

For oral immunization, antigen (TT, FLUVIRAL, or a TT/FLUVIRAL/HBsAg combination), with and without adjuvant, made up to a total volume of 50 μl was administered using a 1 cc tuberculin syringe (Becton Dickinson, Franklin Lakes, NJ) attached to a 20-gauge olive tip steel feeding tube (Fine Science Tools, North Vancouver, BC), which was passed down the oral cavity and esophagus.

For IN immunization, TT alone or with adjuvant, in a total volume of 5–20 μl, was applied as droplets over the external nares of the mouse.

For IR immunization, TT alone or with adjuvant in a total volume of 20 μl was deposited into the rectum via the anus using a 200 μl pipette tip.

2.4.1. Collection of samples

All samples were collected over a two-day period, which was 1 week ± 1 day after the third and final immunization. Plasma and fecal pellets were collected and lung washes carried out as previously described

[21]. Vaginal secretion samples were collected by washing the vaginal cavity three times with 75 μl (225 μl total) of PBS containing 0.1 μg sodium azide (Sigma, St. Louis, MO). Gut washes were obtained by removing the small intestine and passing 200 μl PBS containing 0.1% sodium azide through each of three 10-cm sections. All samples were stored at −20°C until assayed by ELISA.

2.4.2. Evaluation of immune responses

Ag-specific antibodies in the individual samples were detected and quantified by end-point dilution ELISA assay as described previously for IgG, IgG1, IgG2a [12] and IgA [21] isotypes. Titers for IgG isotypes in plasma, and IgA in mucosal samples were defined as the highest sample dilution that resulted in an absorbance value (OD_{450}) at least twice that of non-immune plasma or mucosal sample. Ag-specific antibody titers for a group of animals were expressed as geometric mean titers ± the standard error of the mean (GMT ± SEM) of individual animal values, which were themselves the average of triplicate assays.

2.4.3. Statistical analysis

Data were analyzed using the GraphPAD InStat program (GraphPAD Software, San Diego). The statistical significance of the difference between groups was calculated using transformed data (log_{10}) of ELISA titers, by Student's 2-tailed *t*-test for two groups, or by 1-factor analysis of variance (ANOVA) followed by Tukey's test for three or more groups. Differences were considered to be not significant with $p > 0.05$.

3. Results

3.1. CpG ODN administered with TT at different mucosal sites: Adjuvant effect on plasma IgG

To evaluate the adjuvant activity of CpG ODN at different mucosal sites, TT was administered by three routes of delivery (oral, IR, IN). Without adjuvant, TT induced no or only very low (< 10) levels of anti-TT IgG antibodies in the plasma of mice in the IR or IN groups and low to medium titers in most animals with the oral route (Fig. 1, panels (a–c)). Addition of CpG ODN to TT greatly enhanced the response rate and titers for all routes of delivery. The ranking of anti-TT plasma IgG was oral > IN > IR ($p < 0.05$) with a low dose of CpG (1 μg). At larger doses (10 or 100 μg) equivalent

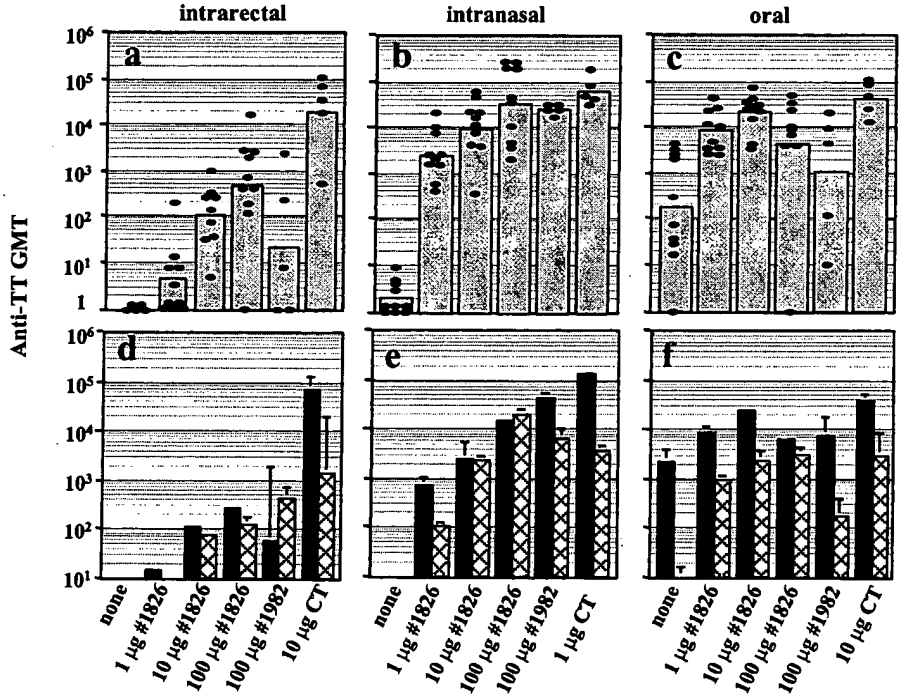

Fig. 1. BALB/c mice were immunized on days 0, 7 and 14 by IR, IN, or oral delivery of 10 μg TT either alone, with a CpG ODN (#1826, 1, 10 or 100 μg), a non-CpG ODN (#1982, 100 μg) or cholera toxin (CT, 1 or 10 μg) as adjuvant. *Upper panels* (a–c): Each point represents the titer for TT-specific antibodies (anti-TT IgG GMT) in individual animals in plasma taken 1 week after final immunization. *Lower panels* (d–f): Each bar represents the group geometric mean (±SEM) of the titer for TT-specific antibodies (anti-TT IgG GMT) of IgG1 (black bars) or IgG2a (hatched bars) isotypes in plasma taken 1 week after final immunization.

responses were seen for oral and IN groups ($p > 0.05$), and these were higher than the IR group (≤ 0.002) (Fig. 1, panels (a–c)). For oral delivery, mean IgG titers were enhanced nearly 100-fold compared to TT alone, with similar effects being obtained with all doses of CpG ODN used (1, 10 or 100 μg, $p > 0.05$) (Fig. 1(c)). With IN delivery, where TT alone resulted in 6/10 with no anti-TT and only 4/10 with very low titer, the addition of CpG ODN gave high titers in all animals (30/30), and there was a significant ODN dose-response ($p < 0.05$) (Fig. 1(b)). The adjuvant effects of CpG ODN were less obvious with the IR route, but there was also a strong dose-response for the ODN with each 10-fold increase in CpG ODN dose giving 10-fold higher anti-TT titers ($p < 0.05$), suggesting that perhaps optimal effects were not realized, even with the 100 μg dose (Fig. 1(a)).

Compared to CT, which was used at commonly reported doses (10 μg for oral and IR, 1 μg for IN), CpG ODN (10 or 100 μg doses) induced equivalent levels of anti-TT IgG for oral and IN routes ($p > 0.05$), but lower levels with the IR route (Fig. 1).

Non-CpG ODN (100 μg dose), which was included to be a negative control, had an unexpected adjuvant effect, with at least some of the mucosal routes. All IN mice (5/5), most oral mice (3/5) but only one IR mouse (1/5) developed good IgG titers (> 1000). This adjuvant effect of non-CpG ODN was unexpected since the same non-CpG ODN (#1982) did not have stimulatory effects on mouse cells in vitro, nor had it been found to have adjuvant effects when given by a parenteral route (intramuscular injection) [12]. A previous IN study had also not detected a non-CpG ODN effect, albeit at a 10-fold lower ODN dose (10 μg) than used in this study [21].

It should be noted that the adjuvant effects of non-CpG ODN on plasma IgG were inferior to those of the same dose of CpG ODN for oral and IR routes. Another difference between the two classes of ODN adjuvants was the Th bias of the IgG response. As an indirect way to determine Th-bias of induced responses, IgG isotypes were measured (Th1: IgG1 < IgG2a, Th2: IgG2a < IgG1). When IgG titers were high enough to detect isotypes (i.e. after oral delivery), TT alone induced a strong Th2 response with virtually no IgG2a. CpG ODN, which has been shown to be a Th1-type adjuvant, was able to overcome the strong Th2 bias of TT and gave a mixed IgG1/IgG2a response with all routes (Fig. 1, panels (d–f)). Furthermore, with IN and oral delivery, the proportion of IgG2a antibodies increased with higher doses of CpG ODN. In contrast, TT-specific antibody responses with both non-CpG ODN (#1982) or CT as adjuvant remained strongly Th2 (IgG1 ≫ IgG2a) biased. Thus, despite the finding that both CpG and non-CpG ODN had an adjuvant effect on TT-specific IgG responses (at the doses tested), the strong Th1 bias typically seen with CpG ODN was not seen with non-CpG ODN.

3.2. CpG ODN administered with TT at different mucosal sites: Adjuvant effect on IgA response

Assays were carried out to detect IgA at mucosal surfaces of the respiratory (lung and nasal wash), digestive (saliva, gut, feces) and reproductive (vagina) systems. After administration of TT alone by IR, IN or oral routes, none or only very low levels of TT-specific IgA were detected in the various mucosal samples recovered from the mice (Fig. 2). In contrast, when CpG ODN was added, it was possible to induce significant levels of IgA in most mice, although this was somewhat dependent upon the route of immunization and dose of CpG ODN. For example, with IR delivery, dose-dependent levels of IgA were detected in the gut wash and feces, but no IgA was found in lung, nasal, vaginal or saliva samples. On the other hand, a TT-specific IgA response was induced at all (6/6) or most (4/6) of the mucosal surfaces tested, with IN or oral delivery respectively. The strength of these IgA responses were dose-dependent for IN but not for oral delivery. Only two of six assays for the IR group had detectable IgA (Fig. 2).

The finding of IgA at multiple mucosal surfaces indicates that the CMIS was activated. Nevertheless, certain surfaces appeared to be preferential for a given route. For example, more mice in the IR group had anti-TT IgA (titers > 5) in the feces (8/30) than the gut (4/30), whereas more mice in the oral group had IgA in gut (16/30) than the feces (5/30). With IN delivery, most mice (11/15) had IgA in nasal washes, but this was not seen after IR or oral immunizations. These results suggest that a stronger mucosal response occurs at the site of antigen uptake than elsewhere (i.e., GALT, RALT and NALT for oral, IR, and IN routes, respectively).

TT-specific IgA titers with CpG ODN were generally as high as or higher than those obtained with CT. Non-CpG ODN was not as potent as CpG ODN for induction of IgA, but it was still possible to obtain significant IgA titers in most mucosal samples after IN, but not IR or oral immunization.

3.3. CpG and non-CpG ODN administered orally with influenza vaccine

Since the non-CpG control ODN (sequence #1982) had an unexpected immunostimulatory effect with mucosal delivery of TT, in the experiments described above, we examined whether similar immunostimulatory effects would also be seen by adding a different non-CpG ODN (sequence #2138) to TT and other

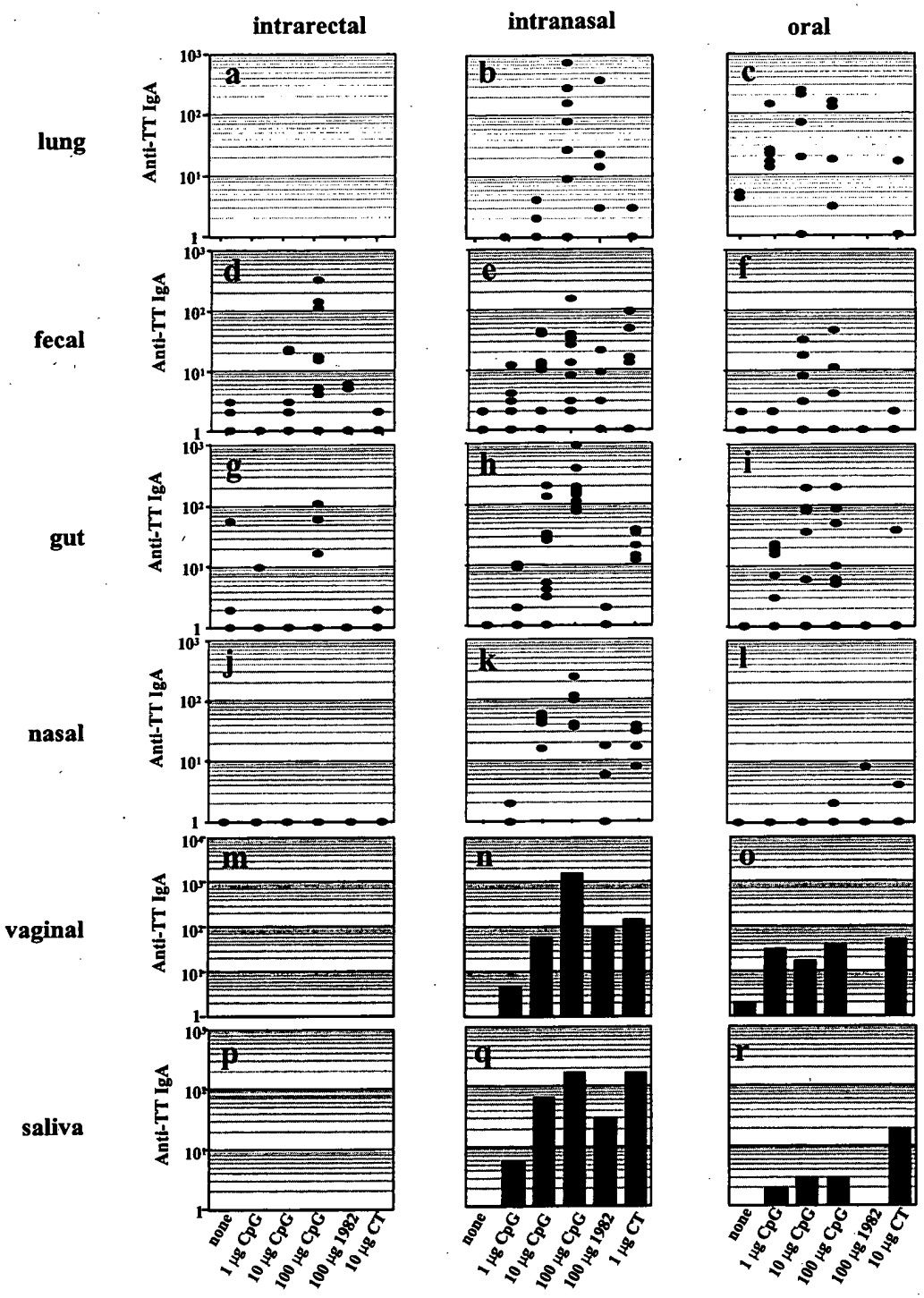

Fig. 2. BALB/c mice were immunized on days 0, 7 and 14 by intrarectal (IR), intranasal (IN), or oral delivery of 10 μg TT either alone, with a CpG ODN (#1826, 1, 10 or 100 μg), a non-CpG ODN (#1982, 100 μg) or cholera toxin (CT, 1 or 10 μg) as adjuvant. Panels (a–c), (d–f), (g–i), (j–l), (m–o), and (p–r) represent TT-specific IgA antibodies obtained in lung, fecal, gut, nasal, vaginal and saliva samples respectively collected 1 week after final immunization from mice immunized using TT as antigen. Each point (panels (a–l)) represents the titer for TT-specific IgA antibodies in individual animals and bars (panels (m–r)) represent the titer for Ag-specific IgA antibodies in pooled samples.

antigens. Sequence #2138 was chosen since it has an identical sequence to CpG ODN #1826 except that CpG is reversed to GpC.

When FLUVIRAL was used as an antigen and was delivered orally without adjuvant, HA-specific plasma IgG had only low titers (< 100) and was predominantly of IgG1 isotype with very little IgG2a, indicating a Th2-type response. With the addition of ODN, titers of plasma HA-specific IgG titers augmented similarly (~5-fold) with CpG ODN (#1826) as well as the non-CpG ODNs (#2138 and #1982) (Fig. 3, upper panel). However, CpG ODN augmented predominantly IgG2a (Th1-like) antibodies, and therefore overcame the strong Th-2 bias of FLUVIRAL alone, whereas the non-CpG ODNs augmented both IgG1 and IgG2a such that the Th2 bias was retained (Fig. 3, bottom panel). Both CpG (#1826) and non-CpG (#2138) ODNs augmented antigen-specific mucosal immunity (IgA) at a number of mucosal sites (lung, vagina and saliva), although IgA titers were higher with CpG than non-CpG ODN (Fig. 4).

3.4. CpG and non-CpG ODN administered orally with a trivalent vaccine

In order to test non-CpG ODN with yet another antigen, HBsAg, as well as to test the adjuvant effects of both CpG and non-CpG ODNs with a multivalent vaccine, mice were immunized orally with a combination of HBsAg/TT/FLUVIRAL alone or with CpG (#1826) or non-CpG (#1982) ODN. When the trivalent vaccine was given without adjuvant, no HBsAg-specific (anti-HBs) IgG was detected in the plasma of mice and mean TT- and HA-specific IgG titers were ~1000 and 100, respectively (Fig. 5). In contrast, when CpG or non-CpG ODN was added, HBsAg-specific IgG was detected and TT- and HA-specific IgG titers were raised ~10- to 20-fold. Despite the similar effects on total IgG, CpG ODN induced more Th1-like responses than did non-CpG ODN, as evidenced by a higher proportion of IgG2a relative to IgG1 for TT- and HA-specific responses (Fig. 6). As seen with single antigens, (Figs. 2 and 4), both CpG and non-CpG ODN were able to augment Ag-specific IgA at a number of mucosal sites (Fig. 7).

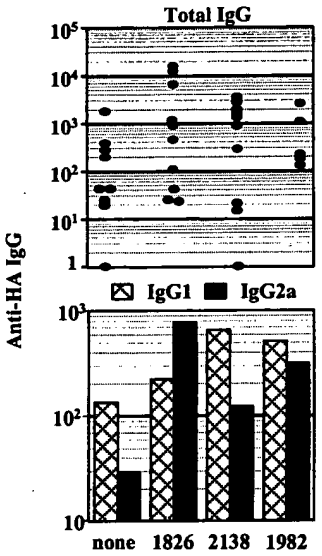

Fig. 3. BALB/c mice were immunized by oral delivery on days 0, 7 and 14 with FLUVIRAL® (50 μl, 1/10 human dose) without adjuvant or in combination with 10 μg of CpG ODN (motif #1826) or non-CpG ODN (motif #2138 or #1982). *Upper panel*: Each point represents the HA-specific IgG titer (anti-HA IgG) for individual samples in plasma taken 1 week after final immunization. *Lower panel*: Each bar represents the group geometric mean of the titer for HA-specific antibodies (anti-HA IgG) of IgG1 (hatched bars) or IgG2a (black bars) isotypes in plasma taken 1 week after final immunization.

4. Discussion

More effective protection against mucosal pathogens could be achieved with mucosal immunization, which induces mucosal as well as systemic immune responses. Furthermore, the existence of a "common mucosal immune system" means that immunization at one mucosal surface can also induce IgA at other mucosal sites [29,30]. Vaccines can be delivered to mucosal surfaces in the form of pills or solutions to be taken orally, nasal drops or sprays, aerosolized solutions that are inhaled, intrarectal or intravaginal suppositories or creams, and eye drops. Mucosal vaccines are also attractive because they are easier and less expensive to deliver, do not need highly trained personnel, have no risk of needle/stick injury or syringe cross-

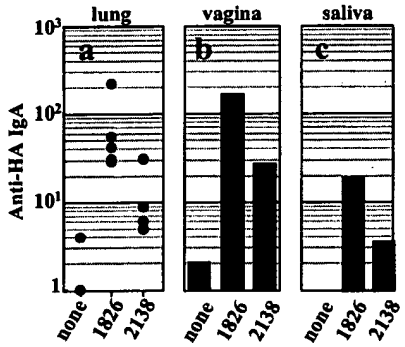

Fig. 4. BALB/c mice were immunized by oral delivery on days 0, 7 and 14 with FLUVIRAL® (50 μl, 1/10 human dose) without adjuvant or in combination with 10 μg of CpG ODN (motif #1826) or non-CpG ODN (motif #2138). Each point (panel (a)) represents the titer for HA-specific IgA antibodies in individual animals and bars (panels (b) and (c)) represent the titer for HA-specific IgA antibodies in pooled samples.

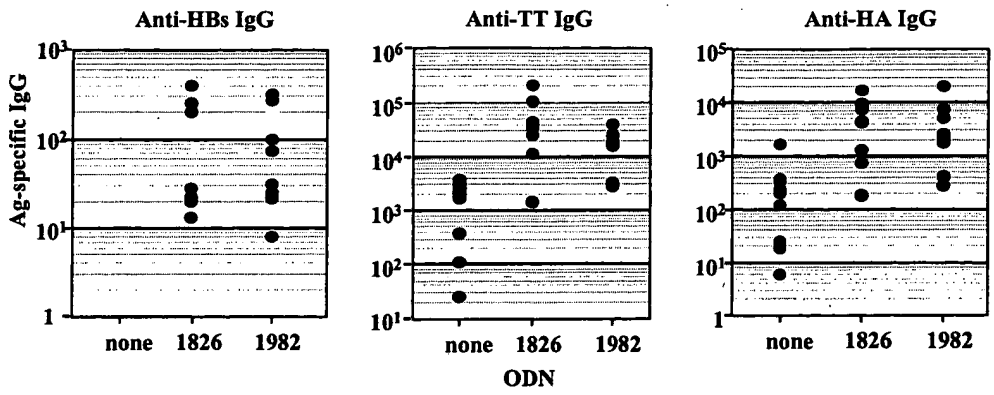

Fig. 5. BALB/c mice were immunized by oral delivery on days 0, 7 and 14 with a combination of HBsAg/TT/FLUVIRAL® (10 μg, 10 μg and 50 μl, respectively) without adjuvant or in combination with 10 μg CpG ODN (motif #1826), or non-CpG ODN (motif #1982). Each symbol represents the titer for HBsAg-specific (left panel), TT-specific (middle panel) or HA-specific (right panel) IgG antibodies in plasma of individual mice taken 1 week after final immunization.

contamination, and may be effective in a broader age range of recipients [31].

Recently, many studies have demonstrated the immunostimulatory properties of CpG ODN with parenteral delivery, and a smaller number with IN delivery [21–24]. However, no comparison has been made using CpG ODN with different mucosal routes. Herein, we have evaluated CpG ODN as a mucosal adjuvant to TT with three routes of mucosal immunization (IN, IR, oral), and also with an influenza vaccine and HBsAg by the oral route. In all cases antigen-specific IgG in plasma was induced as well as secretory IgA, which was found at both local and distant sites, although not all routes induced IgA at all surfaces.

We have found that different mucosal immune profiles are induced when CpG ODN is administered with antigen by different routes. Both IN and oral delivery induced IgA in all six different regional secretions tested whereas, even at high doses, IR delivery did not induce any detectable IgA in lung, nasal, vaginal or saliva samples yet did in gut and fecal samples. In some instances, the strongest mucosal immune re-

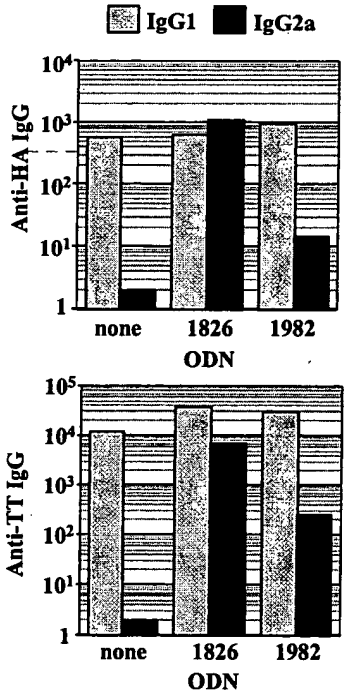

Fig. 6. BALB/c mice were immunized by oral delivery on days 0, 7 and 14 with a combination of HBsAg/TT/FLUVIRAL® (10 μg, 10 μg and 50 μl, respectively) without adjuvant or in combination with 10 μg CpG ODN (motif #1826), or non-CpG ODN (motif #1982). *Upper panel*: Each bar represents the group geometric mean of the titer for HA-specific antibodies of IgG1 (grey bars) or IgG2a (black bars) isotypes in plasma taken 1 week after final immunization. *Lower panel*: Each bar represents the group geometric mean of the titer for TT-specific antibodies of IgG1 (grey bars) or IgG2a (black bars) isotypes in plasma taken 1 week after final immunization.

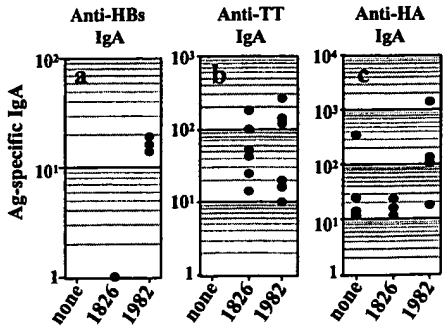

Fig. 7. BALB/c mice were immunized by oral delivery on days 0, 7 and 14 with a combination of HBsAg/TT/FLUVIRAL® (10 μg, 10 μg and 50 μl, respectively) without adjuvant or in combination with 10 μg CpG ODN (motif #1826), or non-CpG ODN (motif #1982). Each symbol represents the titer for HBsAg-specific (panel (a)), TT-specific (panel (b)) and HA-specific (panel (c)) IgA antibodies in lung washes of individual mice taken 1 week after final immunization.

sponses appeared to occur at the site of antigen uptake. For example, only with IN delivery was IgA detected in nasal washes. Furthermore, IgA levels in gut washes were greater with oral than IR delivery, but IgA levels in fecal samples were greater with IR than oral delivery. However, in other cases the pattern was less clear. IN and oral routes of delivery were equivalent for induction of IgA in lung and gut, yet IN was better than oral for induction of IgA in vaginal washes and saliva, and better than IR for gut and fecal IgA. Circulating antibody-secreting cells (ASC) induced by mucosal and systemic immunizations express different sets of adhesion molecules and there is a differential expression of circulating ASC originating from different mucosal sites [32,33]. The distribution of specific antibodies and ASC in the intestine after oral and rectal immunizations may correspond to the vascularization and lymph drainage patterns of the gut [34]. These studies suggest that a certain degree of compartmentalization exists within the CMIS and our findings support this idea. Therefore, vaccination could be optimized by consideration of the route of transmission of the pathogen when choosing the route of vaccine delivery. For example, since the primary sites of transmission of the human immunodeficiency virus (HIV) are the vaginal and rectal mucosae, oral or IN immunization may be more suitable than rectal immunization for protection against HIV on account of the high levels of vaginal and fecal IgA that is induced.

A surprising finding in the present study is that non-CpG ODN were shown to have immunostimulatory effects when delivered to a mucosal surface. Indeed, non-CpG ODN were able to induce equally high levels of Ag-specific plasma IgG as did CpG ODN or CT, a strong mucosal adjuvant conventionally used in animal studies. These findings suggest that, at least at some mucosal surfaces, there is a sequence-independent immunostimulatory activity of ODN, which might possibly be associated with the phosphorothioate backbone of the ODN. The stimulatory effects of non-CpG ODN were totally unexpected since non-CpG ODN do not have such an effect when delivered by a parenteral route (e.g. IM injection) [12], nor do they cause innate immune activation when added in vitro to cultures of murine peripheral blood mononuclear cells [9]. It is possible that the immunostimulatory properties of non-CpG ODN with mucosal but not parenteral delivery are associated with differences in the number and type of cells at the site of antigen uptake. For example, the mucosal epithelium contains a large number of intraepithelial lymphocytes (IELs), which may be the primary source of effector T cells in the mucosal epithelium and play a major role in defending the mucosae [35]. The phenotype markers of IELs at mucosal sites differs from that of peripheral blood lymphocytes. In the adult mouse, 30–50% of IEL carry γδ receptors, compared to only 3% of lamina propria or peripheral lymphocytes [36], and in the human colon epithelium up to 40% of resident lymphocytes have γδ receptors [37]. T cells carrying γδ receptors are believed to play a major role in the primary defence against invading pathogens, and appear to respond in a simpler, more direct manner than the more common αβ T cells. Whereas αβ T cells can only recognize and respond to antigens which have been processed and presented by APCs in conjunction with MHC molecules, γδ T cells can respond directly to MHC proteins without added peptides and also recognize some bacterial antigens without the need for MHC presentation [37]. Since the mucosal surfaces are constantly exposed to a barrage of foreign DNA, it is possible that immune defense mechanisms may have evolved to be less specific and more vigorous than those elsewhere. If so, this could explain, at least in part, why such strong responses were achieved with mucosal but not parenteral delivery of non-CpG ODN.

In the absence of cytokine secretion data, IgG isotypes are commonly used as an indirect means of determining Th-bias of induced immune responses. Using a predominance of IgG2a or IgG1 as an indication of Th1 or Th2 responses respectively, we found that CpG ODN resulted in mixed Th1/Th2 or predominantly Th1-like responses, whether used with single antigens or in a triple antigen combination, whereas non-CpG ODN induced predominantly Th2-like responses (IgG1 ≫ IgG2a) for all antigens. Thus, there appears to be a neutral or Th2 immunostimulatory effect associated with the phosphorothioate backbone, which is shifted to a Th1-like profile when CpG motifs are incorporated. This most likely arises since CpG motifs induce a Th1-like pattern of cytokine production dominated by IL-12 and IFN-γ with little secretion of Th2 cytokines [11]. While Th1-like responses, which are associated with enhanced levels of neutralizing IgG2a antibodies and CTL activity, are desirable for immunization against numerous intracellular viral, bacterial and parasitic pathogens, there are a number of diseases for which Th2 responses may be more important including: organ-specific autoimmune disorders, Crohn's disease, *Helicobacter pylori*-induced peptic ulcer, acute kidney allograft rejection, and unexplained recurrent abortion [38,39]. Thus, it is possible that depending on the nature of the particular disease being treated, there may be a use for a more Th1- or Th2-biased stimulatory ODN as vaccine adjuvant. This is the first report of mucosal application of non-CpG ODN to augment immune responses and is therefore of importance in the development of effective Th2-biased prophylactic or therapeutic strategies. In this study, we have used nuclease-resistant phosphorothioate ODN. It is possible that a

similar immunostimulatory effect will not be seen with phosphodiester ODN, which are rapidly degraded by nucleases; however, we are currently evaluating this possibility.

In summary, mucosal vaccines are desirable to protect against the large number of infectious diseases that are transmitted via mucosal surfaces. Even for some diseases which are not transmitted mucosally but for which global vaccination is desirable (e.g., tetanus, malaria, yellow fever), mucosal vaccines are attractive for large-scale prophylactic vaccination. However, the lack of safe yet effective mucosal adjuvants hinders the development of mucosal vaccines in humans. Our findings that both CpG and non-CpG ODN are effective adjuvants at multiple mucosal surfaces, and that the choice of ODN and route of immunization can influence the nature of induced immune responses which are of considerable interest in the future development of human vaccines against numerous infectious diseases.

Acknowledgements

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CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus.

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Bacterial DNA causes B cell proliferation, immunoglobulin secretion, and Th1-like cytokine secretion, due to unmethylated CpG dinucleotides in particular base contexts (CpG motifs), which are far more common in bacterial DNA than in vertebrate DNA. Synthetic oligodeoxynucleotides (ODN) containing CpG motifs also trigger immune activation, suggesting possible utility as vaccine enhancers. Mice systemically primed with formali inactivated influenza virus mixed with CpG ODN, generated virus-specific serum antibodies at titres approximately seven times higher than mice immunized without CpG; the titres were further increased following an identical second injection. To determine whether CpG could be absorbed through mucosae and enhance vaccination responses, mice were immunized intranasally (IN) with the same preparation of virus with or without CpG ODN or Escherichia coli DNA. Following IN immunization, CpG ODN or E coli DNA promoted increased production of influenza-specific antibodies in serum, saliva and the genital tract, compared with the control groups. These studies indicate that stimulatory CpG ODN are promising new immune enhancers for vaccination applications.

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CpG DNA is a potent enhancer of systemic and mucosal immun responses against hepatitis B surface antigen with intranasal administration to mice.

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Mucosal immunity is difficult to induce with subunit vaccines unless such vaccines are administered with a mucosal adjuvant such as cholera toxin (CT however, CT is toxic in humans. Synthetic oligodeoxynucleotides containing immunostimulatory CpG motifs (CpG) are potent adjuvants for the induction of Th1-like systemic immune responses against parenterally delivered proteins. Here, we show in mice that intranasal delivery of hepatitis B surfac Ag, which alone has no effect, elicits good immune responses when given with CpG oligodeoxynucleotides and/or CT. Overall, CpG is superior to CT for the induction of humoral and cell-mediated systemic immunity as well as mucosal immune responses (IgA) at local (lung) and distant (feces) sites. Furthermore, CpG and CT act synergistically, giving stronger responses than those observed with 10 times more of either adjuvant alone. Ab isotypes wer predominantly IgG1 (Th2-like) with CT, mixed IgG1/IgG2a (Th0) with CpG and predominantly IgG2a (Th1-like) with CpG and CT together.

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